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Identification of Wolbachia like endosymbiont DNA in Setaria digitata genome and phylogenetic analysis of filarial nematodes

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Abstract - Setaria digitata is a Wolbachia-free filarial parasite that causes cerebrospinal nematodiasis in non-permissive hosts such as goats and sheep, leading to substantial economic losses in animal husbandry. Therefore, there arises a considerable need for the development of new interventions to disease control and eradication of this filarial parasite. Owing to the limited knowledge on S. digitata genome, it's host-parasite relationship and the potential impact of the Wolbachia endosymbiont in filarial nematodes, this research was focused on the generation of the draft genome of S. digitata; identification of Wolbachia like endosymbiont DNA in S. digitata genome; phylogenetic analysis as well as functional annotation and metabolic pathway analysis of the genome. A draft genome of 78.8 Mbp size with a GC% of 31.45% was generated for the S. digitata worms collected from the peritoneal cavity of slaughtered cattle, using NGS Illumina platform. FASTA36 sequence similarity analysis was able to identify homologous sequences of coxA and gatB Wolbachia MLST genes within the S. digitata genome, while phylogenetic analysis using Geneious Prime Software revealed that S. digitata is more closely related to the filarial nematodes with Wolbachia endosymbiont than Wolbachia-free filarial nematodes. Furthermore, BLAST2GO analysis was able to identify 6055 annotations and 95 metabolic pathways within the S. digitata genome. Based on FASTA36 and phylogenetic analyses, it can be concluded that ancestors of S. digitata were colonized with Wolbachia in the distant past, and suspected gene transfer may have brought Wolbachia DNA into the S. digitata nuclear genome prior to endosymbiont loss.

Keywords - Setaria digitata, Wolbachia, NGS

Introduction

S. digitata is an ivory-color slimy filarial parasitic-worm with a coiled tapering tail. It is classified under class Secernentea, order Spirurida and family Setariidae. S. digitata resides in the peritoneal cavity of grazing hoofed animals (Shiny et al., 2011; Shin et al., 2002) and cause cerebrospinal nematodiasis, a neuropathological disorder that causes dysfunction of the central nervous system. It leads to lumbar paralysis and eventual death of non-permissive domesticated hosts such as goats, sheep and horses, which cause substantial economic loss in animal husbandry in Asia and the Far East. However, infection of natural hosts such as cattle and buffalos by the S. digitata cause mild disease conditions such as mild fibrinous peritonitis and not considered as parasitic in their natural hosts. Human infections are also reported

causing allergic reactions, eye lesions, abscesses, enlarged lymph nodes and lung inflammation (Taylor and Hoerauf, 1999; Gunawardene and Dassanayake, 2015; Sundar and Souza, 2015)

Most of the filarial nematodes mutually associate with Wolbachia and without Wolbachia, such filarial nematodes cannot survive (Werren et al., 2008). The reason is that the genome of Wolbachia carries the genes required for the metabolism of heam, riboflavin, FAD, glutathione and nucleotides whereas its filarial host does not (Fenn et al., 2006). However, S. digitata does not harbor Wolbachia endosymbiont, but undergo biosynthetic pathways of heam, riboflavin and nucleotides. The genes of those pathways should be present in its genome itself (Voronin et al., 2015). There are two possible explanations for the independent survival of S. digitata. It can be hypothesized that either the ancestors of S. digitata were colonized with Wolbachia in the distant past and horizontal gene transfer (HGT) may have brought Wolbachia DNA into the nuclear genome of S. digitata prior to endosymbiont loss, or the endosymbiotic relationship between Wolbachia and its filarial host is dispensable. Studies showed that Wolbachia free filarial nematode Loa loa evolved own DNA sequences to code heam and riboflavin biosynthesis pathways. For some pathways, they have gained partial gene sequences from Wolbachia, and this indicates the horizontal gene transfer within Loa loa and Wolbachia at some point during the evolution (Desjardins et al., 2013). Therefore, independent survival of S. digitata also can be explained by horizontal gene transfer of Wolbachia gene fragments to the S. digitata genome and/or evolution of their own DNA sequences to code those pathways (Mcnulty et al., 2010). Therefore, identification of Wolbachia like endosymbiont DNA in S. digitata genome and phylogenetic analysis of S. digitata and other filarial nematodes, are important to understand the potential impact of the endosymbiont and to gain insight into the host-parasite relationship.

S. digitata also considered as a model organism for human lymphatic filariasis (HLF), due to their close resemblance to Wuchereria bancrofti, the primary causative agent of HLF, in morphology, histology and antigenic properties (Gunawardene and Dassanayake, 2015). Hence, generation of a draft genome of S. digitata and complete functional analysis and metabolic pathway reconstructions of S. digitata will have a huge impact on the development of novel drugs and/or vaccines for human filariasis and other filarial diseases.

Methodology

Adult worm samples of S. digitata were collected from the peritoneal cavity of cattle (Bos taurus) from the western province. S. digitata worms were washed thoroughly in PBS and preserved in 80% ethanol at −20˚C before analysis. Genomic DNA of adult S. digitata worms was extracted by DNA Micro kit, QIAGEN. Confirmation of extracted S. digitata DNA was done by carrying out a series of PCRs using S. digitata ARV1 specific primers.

After performing quality control (QC), the passed sample was proceeded with the Next Generation Sequencing (NGS) library construction. Sequencing libraries were constructed from the extracted DNA using the TruSeq™ DNA PCR-Free Kit. Purified libraries were loaded into an Illumina HiSeq4000 for paired-end sequencing. Sequenced data (base call files) were converted to raw FASTQ files using the sequencer soon after the initial sequencing. The reads were filtered before assembly such that for a pair of PE reads each read should have more than 90 % of bases with base quality greater than or equal to Q20. Generated 150bp reads were analyzed for K-mers using JELLYFISH. Once the optimum k-

mer size is identified, a de novo draft assembly was built using SOAPdenovo2.

In order to identify Wolbachia like endosymbiont DNA in S. digitata genome, FASTA file containing 2075 S. digitata contigs was blasted against several Wolbachia specific reference sequences such as Wolbachia surface protein (WSP), Wolbachia-specific 16S rRNA and Wolbachia MLST genes (coxA, gatB, fbpA, ftsZ, and hcpA) using FASTA36 program.

Later, using Wolbachia marker sequences (gatB, coxA, ftsZ, fbpA, hcpA, WSP and 16S rRNA), BLASTx was performed against S. digitata, Loa loa, Brugia malayi, W. bancrofti, Onchocerca volvulus and Wolbachia endosymbiont of B. malayi (wBm) protein sequences available on the NCBI non-redundant (NR) protein sequence database. Resulted best hit for each organism for each marker gene was recorded, and the aligned region was obtained. Those aligned sequences of each organism for each marker gene were used to generate a phylogenetic tree using Geneious Prime software. Firstly, sequences were loaded into the program and alignment was done using MUSCLE algorithm and the phylogenetic tree was generated using the Neighbor-joining (NJ) method. Wolbachia endosymbiont of B. malayi (wBm) was used as the out-group.

Lastly, BLAST2GO BASIC software was used for downstream annotations and metabolic pathway reconstruction.

Results and Discussion

After the initial sequencing using the Illumina platform, a total of 97,586,942 reads were obtained with a GC percentage of 31.67% and a Q20 value of 96.09%. Q20 value gives the percentage of bases called that have a quality score of 20 or above. Phred quality score is a numerical value that expresses the accuracy of each nucleotide. Higher the Q number will be higher the accuracy. After the quality control and pre-processing, a total of 79,292,174 reads were obtained with a GC percentage of 31.77% and a Q20 value of 99.18%. A draft genome of 78,774,594 bases belongs to a total of 2,075 contigs was generated after the assembly. The percentage of guanine-cytosine base pairs (GC%) in the assembled draft genome is 31.45%. In a typical random library, it is expected to see a roughly normal distribution of GC content where the central peak corresponds to the overall GC content. An unusually shaped distribution could indicate a contaminated library or some other kinds of a biased subset. Here, the obtained histogram showed a normal distribution and therefore, it can conclude that there is no contamination.

Five Wolbachia marker gene sequences were selected for identification of Wolbachia like endosymbiont DNA in S. digitata genome because of their presence in the Rickettsiales order. These marker genes have a single copy, a wide spatial distribution and a strong stabilizing selection within the Wolbachia genome. Homologous sequences were found only for coxA and gatB. For coxA, homologous sequence was found in the S. digitata contig_633 and for gatB, homologous sequences were found in the S. digitata contig_915 and contig_724, among which contig_915 has the higher similarity. Sequence identity was higher than 50% and e-value was smaller than 10-4, and therefore it can be considered that sequence similarity is accountable.

After performing BLASTx against protein sequences of S. digitata, Loa loa, Brugia malayi, Wuchereria bancrofti, Onchocerca volvulus and wBm available on the NCBI NR protein database using the Wolbachia marker sequences (gatB, coxA, ftsZ, fbpA, hcpA, WSP and 16S rRNA), only coxA gave hits for all six organisms. Therefore, best hit of each organism for coxA was used and aligned using MUSCLE algorithm to create the phylogenetic tree (Figure 1). Neighbor-joining (NJ) method was used here because it is ideal for phylogeny construction from the sequence data, it is rapid, and it does not assume an equal rate of

evolution amongst all lineages. The resulted tree is simply an estimate and is unlikely to represent the true evolutionary tree of these organisms (Figure 2). Resulted tree was a rooted tree with 6 taxa and 11 nodes. The nodes are where lineages diverge, representing a speciation event from a common ancestor. The root node represents the most recent common ancestor of all of the taxa represented on the tree. Wolbachia was used as the outgroup because it has contrasting characteristics relative to the other included taxa. Wolbachia is a bacterium and all other taxa are nematodes. Several clades can be seen in the resulted phylogenetic tree. W. bancrofti and B. malayi forms a clade while O. volvulus and S. digitata form a separate clade. These four taxa all together form a clade that is related to the L. loa, a Wolbachia-free filarial nematode.

According to the evolutionary distance between taxa based on the sum of the branch length (patristic distance), S. digitata is closely related to the O. volvulus, a filarial nematode with Wolbachia endosymbiont while most distance taxon is Wolbachia. It was expected to be closely related to the L. loa since it also a Wolbachia free nematode just like S. digitata. Based on the generated phylogenetic tree, it can be suggested that Wolbachia genes have been laterally transferred into S. digitata genome since S. digitata is more closely related to the filarial nematodes with Wolbachia endosymbiont than Wolbachia-free filarial nematode, and since S. digitata and all other Wolbachia containing nematodes form a clade separately from Wolbachia-free L.loa.

Out of 2075 total contigs put through the BLAST2GO pipeline, 530 contigs did not generate a BLAST hit. Here BLASTx was performed against the NCBI non-redundant database with a cut-off of 1e-3. Out of 1545 BLAST hit generated contigs, 110 contigs generated a BLAST hit without further downstream Gene Ontology (GO) annotation. Remaining 1435 BLAST hit generated contigs were mapped to retrieve GO terms. Out of 1435 mapped contigs, only 1280 contigs generated GO annotations while 155 contigs were only mapped.

Figure 1. Six coxA BLAST hits after alignment using Geneious Software. Source: Geneious Software

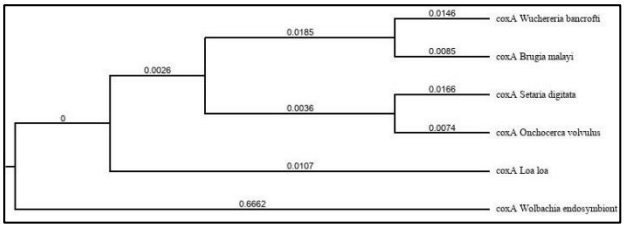

Figure 2. Phylogenetic tree constructed using Neighbour joining method for coxA sequence data. Source: Geneious Software

Majority of the BLAST hits were belong to filarial nematodes with Wolbachia, like Brugia malayi (4998 hits), Wuchereria bancrofti (3311 hits), Brugia pahangi (1940 hits) and Onchocerca ochengi (1673 hits). In here 6055 number of total GO annotations have been done. Overall, 1280 of 1435 (89.1%) mapped BLAST hits were annotated by at least one of the three categories of the GO function classification. The 1280 mapped contigs could be annotated to 6055 GO terms, among which 2297 (grouped in 9 subcategories), 2206 (grouped in 8 subcategories) and 1552 (grouped in 5 subcategories) GO terms could be grouped to the biological process category, molecular function category and the cellular component category, respectively.

As an alternative method of categorizing contigs/sequences by biochemical function, sequences were assigned to biological pathways using the KEGG database. Total of 246 contigs had been clustered into 95 pathways, in which the most over-represented pathways are Biosynthesis of antibiotics (22genes), Phosphatidylinositol signalling

system (11 genes) and Purine metabolism (10genes).

Conclusion

According to the BLAST results of the BLAST2GO functional analysis, the majority of the S. digitata sequences have a higher sequence similarity to Wolbachia containing filarial nematodes like Brugia malayi, Wuchereria bancrofti, Brugia pahangi and Onchocerca ochengi than Wolbachia free filarial nematodes like Loa loa and Onchocerca flexuosa. Based on this result it can be concluded that S. digitata is more closely related to Wolbachia containing nematodes than Wolbachia free nematodes. Phylogenetic analysis also revealed that S. digitata is more closely related to the Wolbachia containing nematodes.

According to the FASTA36 and BLASTx sequence similarity analysis, partial sequences of Wolbachia marker genes (coxA and gatB) were found within the S. digitata genome. This result provided the bioinformatics evidence for the presence of Wolbachia like DNA sequences in the S. digitata genome.

Therefore, based on the BALST results, phylogenetic analysis and the sequence similarity analysis, it can be concluded that as 90% of the filarial nematodes studied up to date contain the Wolbachia, ancestors of S. digitata may have colonized with Wolbachia in the distant past, and HGT may have brought Wolbachia DNA into the nuclear genome prior to endosymbiont loss.

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